

# Single cell microRNA assay

## Absolute quantification of microRNA in single cells using the miRCURY LNA™ microRNA PCR System and the Advalytix AmpliGrid platform.

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**We describe a novel, highly sensitive and specific approach for absolute quantification of microRNA in single cells using the AmpliGrid single cell isolation system combined with the miRCURY LNA™ microRNA PCR System. The method presented here allows for absolute quantification of microRNA copy numbers with single cell resolution, based on standard curve analysis. The combination of the AmpliGrid system and the miRCURY LNA™ microRNA PCR System yields highly reproducible data with excellent PCR assay efficiency and low assay variation between cells. This single cell microRNA expression method enables researchers to effectively analyze microRNA expression in sub-populations of cells instead of having to rely on average expression values of a large heterogeneous cell population.**

### Introduction

MicroRNAs are small, non-coding RNA molecules (~22 nucleotides) that regulate expression of genes involved in many biological processes, such as development, differentiation, morphogenesis and apoptosis<sup>1</sup>. MicroRNAs have also been implicated in a number of pathological conditions involving the cardiovascular system and central nervous system, as well as in numerous cancers, where they act as oncogenes or tumor suppressor genes<sup>2-4</sup>. A large field of microRNA research has emerged since this class of molecules was described in 2001<sup>5-7</sup>, but their very small size has made it difficult to detect microRNA specifically and sensitively using standard molecular biological methods.

Locked Nucleic Acids (LNA™) are a class of nucleotide analogs which, when incorporated into a DNA oligonucleotide, enhance the hybridization properties and affinity of the oligonucleotide to a complementary DNA or RNA strand. The advantages of incorporating LNA™ into DNA oligonucleotides in order to enhance the sensitivity and specificity of microarray probes, Northern blot probes and ISH probes for microRNA analysis, have been established in a number of scientific papers<sup>8-10</sup>. In addition, the use of LNA™ in PCR primers has allowed the development of highly sensitive microRNA real-time PCR assays that enable single base discrimination between closely related microRNA family members<sup>11-13</sup>. Traditional gene expression experiments characterize averages of thousands to millions of cells from

a particular tissue or cultured cell population. However, apparently homogenous cell populations can contain a large diversity of cellular phenotypes, and sometimes researchers are interested in analyzing only a subpopulation of the cells<sup>14</sup> or even single cells. To investigate such subpopulations, it is necessary to apply sensitive and specific single cell isolation and analysis methods.

The AmpliGrid platform ([www.advalytix.com/ampligrid](http://www.advalytix.com/ampligrid)) is based on a chemically structured microscope slide which is divided into 48 reaction sites designed for ultra-low-volume reactions (figure 1). When combined with cell sorting methods (e.g. flow cytometry sorting, laser microdissection and micromanipulation) this innovative system enables efficient and reproducible single cell analysis, low copy number approaches and ultralow-volume enzymatic reaction techniques (e.g. reverse transcription and PCR amplification)<sup>15-16</sup>.

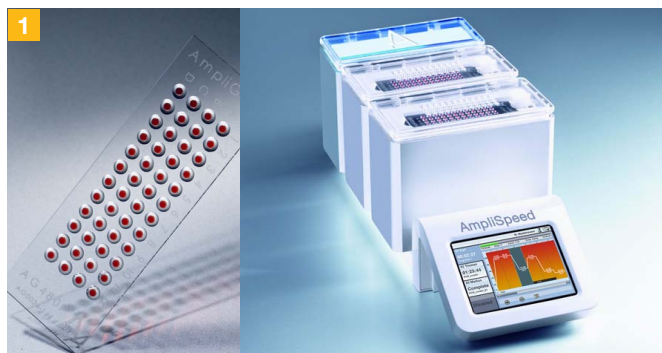


Fig. 1: AmpliGrid AG480F slide and AmpliSpeed ASC200D slide cycler

Here, we describe how to perform an absolute quantification of microRNAs in single HEK293 cells by combining the AmpliGrid™ platform and the miRCURY LNA™ microRNA PCR System from Exiqon ([www.exiqon.com/ls](http://www.exiqon.com/ls)).

### Experimental approach

The process of single cell microRNA expression analysis can be divided into six major steps.

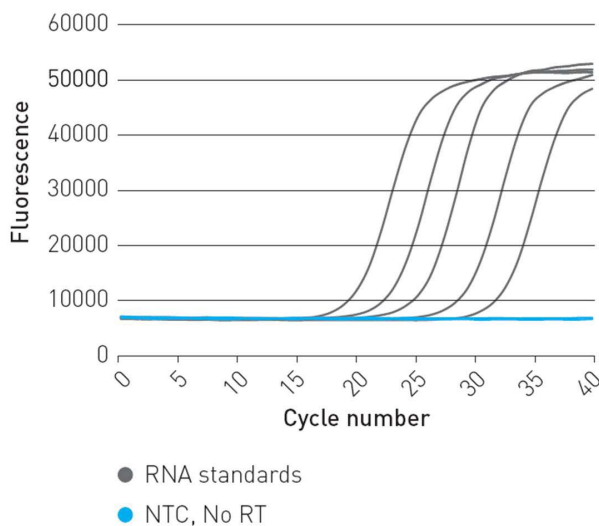
- 1) Cell growth/isolation
- 2) Deposition of individual cells onto the AmpliGrid slide
- 3) Desiccation of cells
- 4) First-strand cDNA synthesis
- 5) Real-time PCR
- 6) Data analysis.

For this experiment we used human embryonic kidney (HEK 293) cells cultured using standard protocols. Individual HEK 293 cells were deposited into separate reaction sites on AmpliGrid AG480F slides by micromanipulation. Correct deposition of the cells was quality assured by optical inspection. The cells were dried at room temperature and low volume (1  $\mu$ L/reaction site) first-strand cDNA synthesis was performed on the reaction sites according to a modified protocol (available at [www.exiqon.com/microRNA-pcr-primer](http://www.exiqon.com/microRNA-pcr-primer) and

[www.advalytix.com/protocols](http://www.advalytix.com/protocols)) using the miRCURY LNA™ first-strand cDNA kit and the microRNA-specific RT primers for hsa-miR-17b and hsa-miR-19 from the miRCURY LNA™ microRNA primer sets. First-strand reactions containing negative control samples and synthetic RNA samples (for a standard curve ranging from 0.1fM to 1pM) were also performed on separate reaction sites. All reactions were covered with 5  $\mu$ L sealing solution and placed on an AmpliSpeed slide cycler ([www.advalytix.com/amplispeed](http://www.advalytix.com/amplispeed)) for thermal treatment (figure 1).

Each cDNA was diluted 1:2.5 (2.5  $\mu$ L total volume) in nuclease-free water and 1  $\mu$ L was transferred to a PCR tube. Subsequently, real-time PCR was performed using miRCURY LNA™ SYBR® Green master mix and microRNA-specific PCR primers. The reactions were run on a real-time PCR thermal cycler according to the standard protocol. Data analysis included melting curve quality control, generation of standard curves (figure 2) for absolute quantification of the microRNA in HEK293 cells, and calculation of assay efficiencies for individual cells.

**2** Figure 2: Sensitivity of the miRCURY LNA™ microRNA PCR System. Amplification curves for a serial dilution ( $6 \times 10^2$ – $6 \times 10^6$  RNA copies) of a synthetic hsa-miR-19 standard are shown. NTC; no template control, No RT; no reverse transcriptase. The mean assay efficiency for hsa-miR-19 was 1.939 (SD $\pm$ 0.028) (not shown).

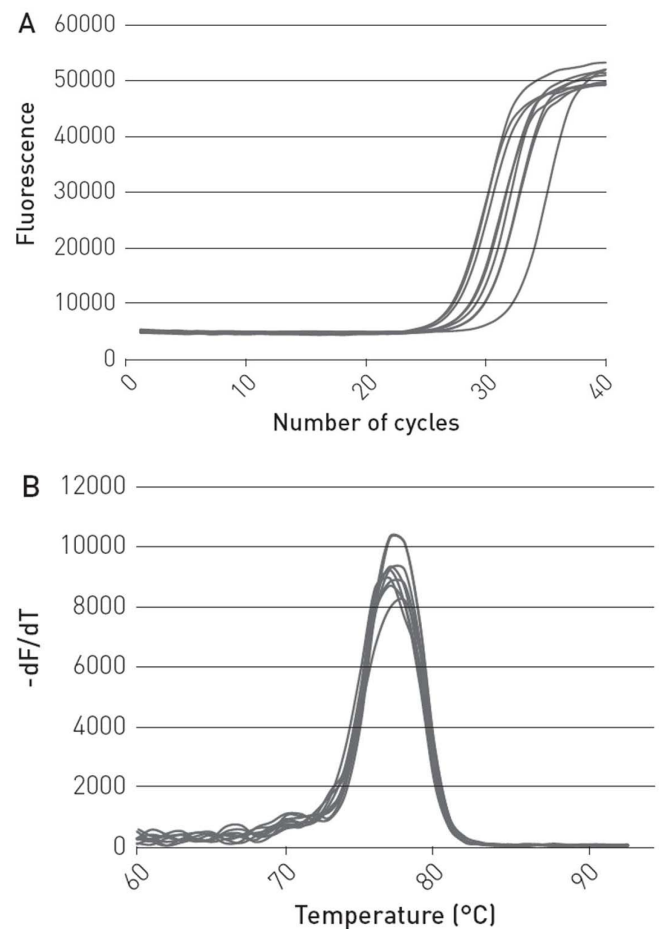


## Results and discussion

The combination of the AmpliGrid single cell PCR System and the miRCURY LNA™ microRNA PCR System enables researchers to perform ultra-low-volume RT reactions

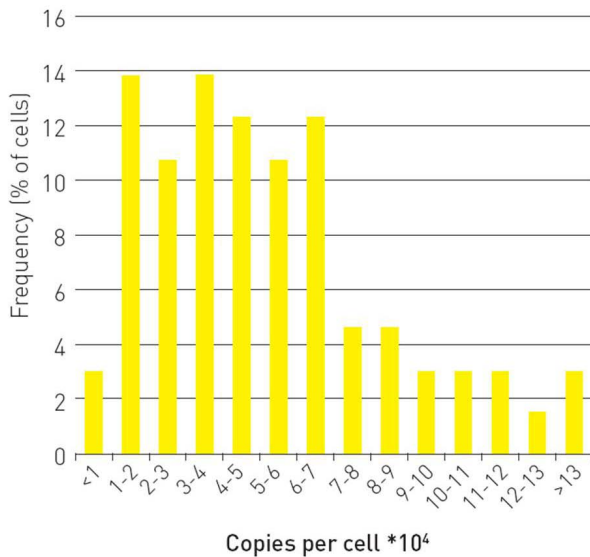
followed by real-time PCR standard curve analysis and subsequent absolute quantification from single cells in a simple and convenient manner. The isolated single cells were spotted onto the AmpliGrid slide before the first-strand cDNA synthesis, resulting in further improved assay efficiency and highly reproducible data (figure 3).

**3** Figure 3: Specific and reproducible amplification of hsa-miR-19 in single cells. Amplification (A) and melting (B) curves resulting from PCR amplification of hsa-miR-19 in 9 different single cell reactions are shown as examples. Overall the melting curves show a major peak corresponding to a  $T_m$  of 77°C. The average assay efficiency for hsa-miR-19 on the 78 single cells interrogated was calculated to be 1.927 (SD $\pm$ 0.040).

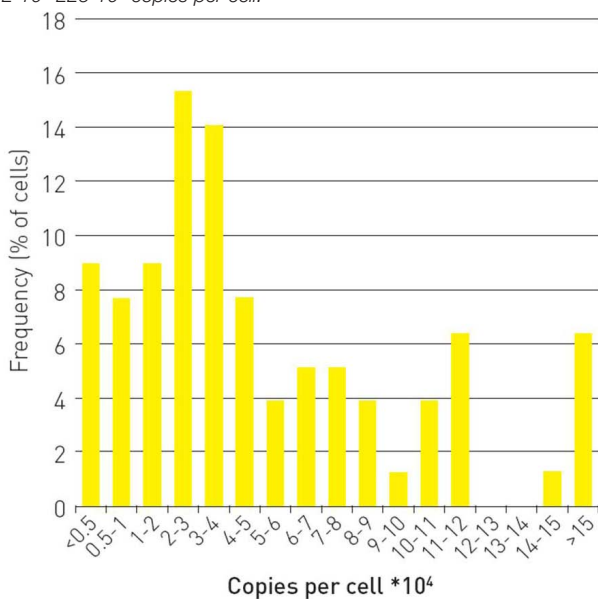


Real-time PCR of hsa-miR-17b and hsa-miR-19 from individual cells resulted in typical amplification curves and the first derivative of the melting curves showed only one major  $T_m$  peak corresponding to the well-defined melting temperature ( $T_m$ ) of the amplicons (figure 3). The average assay efficiencies of the hsa-miR-17b and hsa-miR-19 assays were 1.947 (SD $\pm$ 0.036) and 1.927 (SD $\pm$ 0.040), respectively, which is in accordance with the high sensitivity and specificity of the two assays. The minimal standard deviations of the assay efficiency demonstrated limited cell-to-cell assay variation and excellent reproducibility of the two microRNA assays on the AmpliGrid platform.

**4** Figure 4: Distribution of hsa-miR-17b copy number in HEK293 cells. Absolute quantification of hsa-miR-17b, using the AmpliGrid™ and miRCURY LNA™ microRNA PCR Systems, was based on standard curve analysis. The average assay efficiency was 1.947 (SD±0.036). According to the standard curve calculations, hsa-miR-17b ranged from  $9 \times 10^3$ –  $15 \times 10^3$  copies per cell.



**5** Figure 5: Distribution of hsa-miR-19 copy number in HEK293 cells. Absolute quantification of hsa-miR-19, using the AmpliGrid™ and miRCURY LNA™ microRNA PCR Systems, was based on standard curve analysis. The average assay efficiency was 1.927 (SD±0.040). According to the standard curve calculations, hsa-miR-19 ranged from  $2 \times 10^3$ –  $225 \times 10^3$  copies per cell.



The distribution of the hsa-miR-17b and hsa-miR-19 copy numbers in cells are shown in (figure 4 and 5). The copy numbers of hsa-miR-17b and hsa-miR-19 in the 78 analyzed cells fall within a 20-fold and 100-fold range, respectively. However, in over 70% of the cells the copy numbers for both microRNAs range between 7000 and 70 000. This relatively high copy number range is not surprising since we picked two well-expressed microRNAs (according to the

Mammalian MicroRNA Expression Atlas<sup>18</sup>) for the analysis. In addition, the copy number ranges are in accordance with other published observations from single cell microRNA expression analyses<sup>19</sup>. The differences in expression between cells are most likely due to physical differences, e.g. cell cycle status, which explain the heterogeneous nature of cell-line populations, despite similar phenotype and morphology, as well as growth conditions. This study did not assess other important physical factors (such as receptor status or developmental stage of the embryonic cell) that can be used to cluster cells based on their characteristics<sup>20</sup>. However, the possibility of combining physical characterization (e.g. receptor status) and molecular characterization (e.g. microRNA expression analysis) of single cells is likely to be of great value in areas such as cancer and stem cell research.

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